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Genetic Characterization of Critically Endangered *Puntius sarana* (Hamilton) and the Exotic *Barbonymus gonionotus* (Bleeker) (Cyprinidae: Cypriniformes) by DNA Fingerprinting

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ABSTRACT:
Randomly amplified polymorphic DNA (RAPD) markers were used to elucidate the genetic structure of three stocks of critically endangered indigenous olive barb, *Puntius sarana* and one stock of exotic Thai silver barb *Barbonymus gonionotus* in Bangladesh. Five selected random decamer primers were used to amplify the RAPD markers of 20 fishes from each stock. The primers yielded a total of 48 RAPD bands of which 43 were considered as polymorphic (P_{95}). All the stocks showed almost similar level of intra-stock similarity indices. The population differentiation value (PhiPT = 0.698) between the indigenous and exotic barb was found to be highly significant (P<0.001). The UPGMA dendrogram showed two distinct clusters. The *B. gonionotus* alone made one cluster, and the remaining three *P. sarana* stocks made another cluster. The results showed significant genetic differences between the two species. Among the five primers, OPA 17 can be used as species-diagnostic markers to distinguish the two species. The RAPD technique has been found to be an efficient tool for genetic characterization of the two barb species to provide information on their genetic stock structure.

Key words: Genetic Characterization, Critically Endangered, *Puntius sarana*, Exotic, *Barbonymus gonionotus*, RAPD Markers, Polymorphic loci.

INTRODUCTION

Puntius sarana (Hamilton) belonging to the group of barb of the family Cyprinidae is indigenous in Bangladesh. It is the largest barb available in the Indian sub-continent and one of the popular food fishes having commercial importance in Bangladesh. *P. sarana* attains a maximum length of 42 cm and a weight of 1.5 kg showing great potential as an aquaculture species. Induced breeding protocol of *P. sarana* has already been developed in hatchery conditions [1]. In the past, this freshwater barb was abundantly available in the floodplains and rivers of Bangladesh. Presently, the availability of this fish has declined at a level that it is now considered as a critically endangered species [2] needing immediate measures for protection and conservation. The decline in abundance has been attributed to indiscriminate fishing of broodstocks and juveniles, habitat degradation, aquatic pollution and population pressures on resources. It is thus essential to reveal if the decline in availability of the fish in nature has made any signature on the genetic constitution of the stocks and to develop effective conservation strategy of the germplasm.

As a country of wetlands, Bangladesh is very rich in fish diversity. Even then as many as 24 species of fish have been introduced into Bangladesh for aquaculture and ornamental purposes. Thai silver barb, *Barbonymus gonionotus* (Bleeker) was brought in 1977 from Thailand. It is an inhabitant of the Southeast Asian river systems. Now-a-days *B. gonionotus* is cultured in Bangladesh.

The Randomly Amplified Polymorphic DNA (RAPD) fingerprinting technique allows detection of polymorphisms by randomly amplifying independent multiple regions of the genome through PCR using single arbitrary primers [3, 4]. RAPD fingerprinting technique is simple, fast and sensitive and allows the examination of genomic variation without prior knowledge of DNA sequences. RAPD-PCR has been widely employed in fisheries studies.

Genetic variation enables a species or a stock to adapt to changes in their environment. Genetic data are needed to understand the structure of stocks so that appropriate decisions can be made towards conservation of the species [5]. The indigenous barb *P. sarana* has a great prospect as an aquaculture species in Bangladesh. Therefore, we need to obtain adequate information on its population genetic structure, which is very important for its management. We report here the genetic constitution of three stocks of the indigenous barb *P. sarana* and one stock of exotic Thai barb *B. gonionotus*. We also report the diagnostic RAPD markers for the two barb species.

MATERIALS AND METHODS

Collection of fish samples and isolation of genomic DNA

Three stocks of indigenous olive barb *P. sarana* were selected for the study**:** two wild stocks (one from Chalan *beel* - under Natore district and one from the river Mogra - under Netrokona district) and one captive stock from a private hatchery- Brahmmaputra

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Fish Seed & Hatchery Complex, Mymensingh, Bangladesh during August-September, 2008. The hatchery samples were three months old and the hatchery operator collected broods from a *haor* (large natural depression) of Sunamgonj district. The samples were the first generation of that wild broods reared in this private hatchery. For comparing the genetic variability, one sample of exotic silver barb *B. gonionotus* was collected from the same hatchery. In order to perform DNA fingerprinting analysis, 20 fish were randomly taken from each stock.

Tissue samples were clipped from the caudal fin of each fish using scissors and forceps and immediately preserved in individual microfuge tube containing 95% ethanol and stored at -20°C. For isolation of DNA, approximately 30 mg of fin tissues from each sample was cut into small pieces and ground with a tissue grinder in 1.5 ml microfuge tube. The genomic DNA was isolated following cell rupture and proteinase-K digestion, phenol: chloroform: isoamyl alcohol (25:24:1; v/v/v) extraction, and ethanol precipitation as described by Islam and Alam [6]. All DNA samples were tested qualitatively (degradation of DNA) using 1% agarose gel and quantified using a photometer (Biophotometer plus, Eppendorf, Germany).

Primer Selection

Forty decamer primers from two kits (20 from kit A and 20 from kit B) of random sequence (Operon Technologies, Inc., Alameda, CA, USA) were screened on the sub-samples of two randomly chosen indigenous barb and Thai barb from each of the stocks to test their suitability for amplifying RAPDs that could be accurately scored. Primers were evaluated on the basis of intensity of bands and consistency within individuals and potential of differences in stocks (polymorphisms). Among them a final subset of five primers exhibiting highest quality banding patterns and sufficient variability were selected for analysis of 20 random samples from each stock.

PCR amplification and visualization of amplified products

The amplification conditions were based on [3], with some modifications. PCR reactions were performed on each DNA in a 10 µl reaction mix containing 1 µl of 10× *Taq* polymerase buffer, 2.0 µM of primer, 0.25 mM of each dNTPs, 1 unit of *Taq* DNA polymerase (GENEI, Bangalore, India) and 100 ng of genomic DNA. DNA amplification was performed using a gradient thermal cycler (Master Cycler Gradient, Eppendorf, Germany). The reaction mix was preheated at 94ºC for 3 min followed by 40 cycles consisting of 30 seconds denaturation at 94ºC, 1 min annealing at 38ºC and 2 min elongation at 72ºC. After the last cycle, a final step of 7 min at 72ºC (final extension) was added to allow complete extension of all amplified fragments.

The amplified products from each sample were separated electrophoretically on 1.4% agarose gel containing ethidium bromide. Two molecular weight DNA markers (Lambda DNA/EcoRI/HindIII digest and 100bp ladder) were electrophoresed alongside the RAPD-PCR products. DNA bands were observed on GelDoc system and image was saved in a computer.

Analysis of RAPD data

In order to focus on some salient statistical issues, two assumptions were made for the analysis of the RAPD data. First assumption was that the interpretation of banding patterns on gels should be accomplished in a completely unambiguous manner. Second assumption was that each locus should be treated as a two-allele system. Each band was assumed to represent the dominant genotype at the locus, whereas lack of the same band in another individual was assumed to correspond to the alternative homozygous recessive genotype in the Hardy–Weinberg equilibrium [7].

RAPD patterns were visually analyzed and scored from photographs. For the analysis and comparison of the patterns, distinct and well-separated bands were scored. All distinct bands or fragments (RAPD markers) were given identification numbers according to size and the RAPD markers were determined by recording the presence (1) or absence (0) of these bands for each fish and each primer separately, neglecting other weak and unresolved bands. For more accuracy, band scoring was performed by two persons independently.

The scores obtained using all primers in the RAPD analysis were pooled for constructing a single data matrix. This was used to estimate gene frequency, polymorphic loci, Nei's [8] gene diversity, gene flow (Nm), genetic distance (D) and to construct an unweighted pair group method of arithmetic mean (UPGMA) dendrogram among stocks with 1,000 simulated samples using the POPGENE (Version 1.31) program [9]. The sizes of the RAPD markers were estimated by using the software DNAfrag (Version 3.03) [10]. Analysis of Molecular Variance (AMOVA) was performed using the software GenAlEx version 6.1 [11].

The similarity index values (SI) between the RAPD profiles of any two individuals on the same gel were calculated from RAPD markers of the same molecular weight on the data matrix according to the following formula given by Nei and Li [12]:

$$\text{Similarity index (SI)} = 2N_{AB}/ (N_A + N_B) \qquad \text{(Eqn. 1)}$$

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where, N_{AB} is the total number of RAPD bands shared by individuals A and B, and N_A and N_B are the total number of bands produced by individual A and B, respectively. Thus, genetic-similarity reflects the proportion of bands shared between two individuals and ranges from 0 (no common bands) to 1 (all bands identical).

RESULTS

RAPD polymorphisms

Among the 40 initially tested primers, five (OPA12, OPA17, OPB03, OPB07 and OPB20) yielded comparatively large number of bands with good resolution. The primers produced different RAPD patterns and the number of fragments amplified per primer varied. The five primers yielded a total of 48 reproducible and consistently scorable RAPD bands of which 43 (89.58%) were considered as polymorphic (P_{95}). The number of bands per primer ranged from 9 to 11. The overall polymorphism produced by primer OPB07 was the lowest (80%) while primers OPB03 and OPB20 produced 100% polymorphism (Table 1).

A total of 30 bands in the two species have been detected as private bands: 12 in *P. sarana* and 18 in *B. gonionotus.* These bands were absent in one species and present in most but not all individuals of the other species analyzed. In addition to these, six species diagnostic bands, three present in all the samples of *P. sarana* but absent in *B. gonionotus* and three present in all samples of *B. gonionotus* but absent in *P. sarana* were also detected. Primer OPA 17 produced a consistent band of 703bp in all the stocks of indigenous barb and a band of 387bp in all the samples of Thai barb (Figure 1).

Genetic variability parameters

The proportion of polymorphic loci in the hatchery stock (43.34%) was higher than those of the other indigenous and Thai barb stocks (Table 2). Like polymorphic loci, other genetic variability parameters such as observed and effective number of alleles were also found to be higher in the hatchery sample of the local barb. The within-stock gene diversity was found to be the highest in the hatchery stock of the indigenous barb (0.1619±0.2013) followed by the Chalan *beel* (0.1449±0.1869) and Mogra river stock of indigenous barb (0.1439±0.2051) and the hatchery stock of Thai barb (0.086±0.1684).

The value of intra-stock similarity index (SI) was the highest (94.26%) in the Thai barb sample and lowest (87.25%) in the hatchery stock of the indigenous barb. Among the indigenous barb stocks, the intra-stock SI was the highest in the Mogra river stock.

Table 1 - RAPD primers with corresponding bands amplified by PCR using DNA samples of *P. sarana* and *B. gonionotus*

Primers	Sequence (5´-3´)	Scorable bands	Size range (bp)	Polymorphic bands	Polymorphism (%)
OPA12	TCGGCGATAG	11	200-8699	9	81.82
OPA17	GACCGCTTGT	9	311-1456	8	88.89
OPB03	CATCCCCCTG	9	282-3776	9	100.00
OPB07	GGTGACGCAG	10	219-2252	8	80.00
OPB20	GGACCCTTAC	9	227-2142	9	100.00
Total		48		43	89.58

Table 2 - Number and proportion of polymorphic loci, gene diversity and Similarity index for the studied *P. sarana* and *B. gonionotus* stocks

Stock	No. of Polymorphic loci	Percentage of Polymorphic loci	Observed No. of alleles	Effective No. of alleles	Gene diversity (Mean±SD)	Similarity Index
HPs	18	43.34	1.4333±0.50	1.2802±0.37	0.1619±0.20	87.25
CPs	16	41.27	1.4127±0.49	1.2408±0.33	0.1449±0.19	90.13
MPs	15	36.35	1.3635±0.49	1.2568±0.38	0.1439±0.21	91.34
HBg	9	20.51	1.2443±0.39	1.1955±0.34	0.086±0.17	94.26
Overall	43	89.58	1.8942±0.31	1.5402±0.28	0.3600±0.12	90.75

Note: HPs = Hatchery *P. sarana*, CPs = Chalan *beel P. sarana,*
MPs = Mogra *P. sarana*, HBg = Hatchery *B. gonionotus*.

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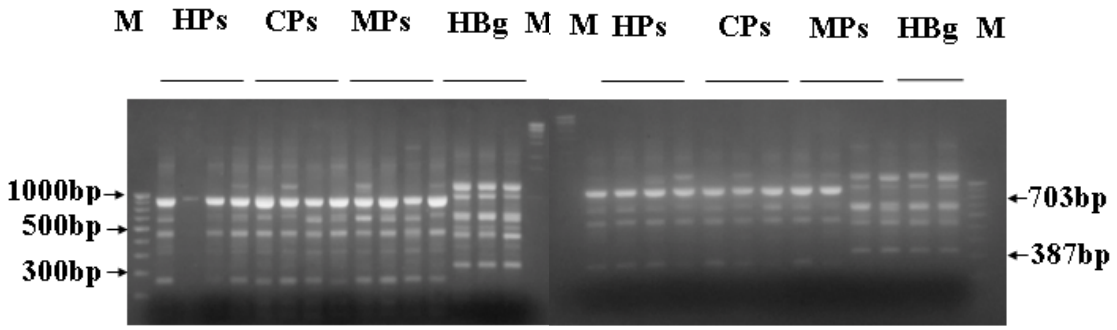

Figure 1 - Random amplified polymorphic DNA (RAPD) profiles of *P. sarana* and *B. gonionotus* for the 'species-diagnostic' primer OPA 17 of the Operon kit A. M: Molecular weight markers (Lambda DNA/EcoRI/HindIII digest and 100bp DNA ladder); HPs: Hatchery *P. sarana*, CPs: Chalan *beel P. sarana*, MPs: Mogra *P. sarana*, HBg: Hatchery *B. gonionotus*. The species diagnostic bands of 703bp for *P. sarana* and 387bp for *B. gonionotus* are shown by arrow heads.

Overall Genetic Variations

Overall gene diversity across all primers for all loci was 0.3600 ± 0.1248 (Table 2). Partitioning of RAPD variance within and among stocks was performed using the AMOVA procedure. The percentages of molecular variance among and within stocks of indigenous and Thai barb were 70% and 30% respectively. However, percentages of molecular variance among and within stocks of the indigenous barb stocks were 10% and 90% respectively. The population differentiation value (PhiPT = 0.698) obtained among the indigenous and Thai barb was found to be highly significant whereas the value (PhiPT = 0.09) among the three indigenous barb stocks was found to be insignificant (Table 3).

Genetic Identity and Genetic Distances

The values for inter–stock genetic identity ranged from 0.4046 to 0.9699 for the four stocks. The genetic identity between the Chalan *beel* and Mogra river stock of the indigenous barb was the highest while that between the river Mogra and the Thai barb stock was the lowest (Table 4).

The genetic distances between the pairs of stocks ranged from 0.0308 to 1.0418. The genetic distance between the river Mogra and Chalan *beel* stock of the indigenous barb was the lowest while that between the river Mogra and the Thai barb stock was the highest (Table 4). The UPGMA dendrogram based on Nei's [13] original measures of genetic distance (D) resulted in two major clusters. The Thai barb alone made one cluster and the three stocks of the indigenous barb made another cluster (Figure 2).

Table 3 - Estimates of genetic variations

	Gene flow	Among stock variation	Within stock variation	Population differentiation PhiPT
Overall	0.32	70	30	0.698**
Local barb	3.44	10	90	0.09 (NS)

Table 4 - Genetic identity (above diagonal) and genetic distance (below diagonal) among different stocks of *P. sarana* and *B. gonionotus* (Nei's original measure of genetic identity and genetic distance, 1972)

Stock ID	HPs	CPs	MPs	HBg
HPs	****	0.9603	0.9562	0.4355
CPs	0.0408	****	0.9699	0.4127
MPs	0.0450	0.0308	****	0.4046
HBg	0.9442	1.0023	1.0418	****

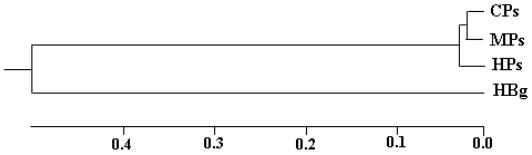

Figure 2 - UPGMA dendrogram based on Nei's (1972) genetic distance showing genetic relationship among stocks of *P. sarana* and *B. gonionotus*; HPs = Hatchery *P. sarana*, CPs = Chalan *beel P. sarana*, MPs = Mogra *P. sarana*, HBg = Hatchery *B. gonionotus*.

DISCUSSION

Genotypic profiles of DNA fingerprints by RAPD markers

The RAPD method is one such technique that has attracted widespread interests. However, genetic characterization with RAPD marker has been hampered by the lack of complete genotypic information due to its dominant nature [7]. Generally, the number and size of the fragments generated depend upon the nucleotide sequence of the primers used and the source of the template DNA, resulting in the genome specific fingerprints of random DNA fragments. Analysis of five primers detected different levels of genetic diversity in each of the stocks studied. The results of the present study demonstrated a great genetic difference between *P. sarana* and *B. gonionotus*, though they are closely related species. Some species specific loci have been detected that can easily distinguish the two barb species. A total of 6 bands can be marked as 'species-diagnostic markers': three in *P. sarana* and three in *B. gonionotus*. These bands were present in all individuals of the respective species analyzed and absent in the other species. Primer OPA 17 can be considered as a species-

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diagnostic marker because it produced a consistent band of 703bp in all the *P. sarana* samples and a consistent band of 387bp in all the Thai barb samples. In this study, a total of 30 bands in the two species have been detected as private bands: 12 in *P. sarana* and 18 in *B. gonionotus.* They can be termed as 'species-exclusive markers' because these were absent in one species but gave a consistent band of respective base pair in most but not all the individuals of the other species. The presence of a high percentage of unique bands is presumably due to the large amount of sequence differences between the genomes [4]. It is also further predicted that the DNA fragments shared by two closely related individuals of a species are allelic while for inter-species comparison, the fragments of equal sizes may have originated from a non-allelic genomic region [14]. The use of many RAPD primers may provide large number of species-specific RAPD markers for the analysis [15].

The percentage of polymorphic loci was found to be higher in the hatchery stock of indigenous barb (43.34%) which was more or less similar with the average (45%) polymorphic loci found in four Indian major carps (*Labeo rohita, Catla catla, Labeo calbasu* and *Cirrhinus mrigala*) [16], that is indicative of relatively higher level of genetic variation. Like the percentage of polymorphic loci, other measurements such as gene diversity (0.1619 ± 0.2013), the observed number of alleles (1.4333 ± 0.5008) and the effective number of alleles (1.2802 ± 0.3682) were also found to be higher in the hatchery stock of the indigenous barb compared to other stocks. In the present study, hatchery stock of the indigenous barb showed higher level of genetic variation than the two wild sources. It can be noted here that the broods of indigenous barb used in the hatchery were collected from a *haor*(large natural depression) of Sunamgonj district and the samples used in the present study were the first generation of that wild stock. Therefore, it can also be considered as a wild stock. The indigenous barb is yet to be popularized for large scale production among the hatcheries and it is a relatively new introduction in the studied hatchery. Therefore, the effect of captive management in the hatchery was not apparent in the hatchery stock of local barb.

The percentage of polymorphic loci in the Thai barb (20.51%) was found to be lower than those of the indigenous barb stocks. A higher level of intra-species similarity index (94.26) and lower number of polymorphic loci (9) and gene diversity (0.086±0.1684) in *B. gonionotus* reflect a relatively lower level of genetic variability in this species compared to *P. sarana*. This may be attributed to the maintenance of a limited number of individuals introduced in Bangladesh from Thailand and their repeated propagation over a long period in the hatcheries. The similarity index - the average fraction of shared fragments provides a sensitive indicator of relative levels of stock homozygosity [17]. Thus, stocks having higher similarity index are more homogenous groups [18]. The intra-specific similarity index in hatchery stock was found to be higher than those of wild stocks of a particular species of the Indian major carps [16, 6, 19]. Genetic drift may also increase the level of within stock similarity index, since there is less or no chance of representation of the rare alleles in such cases.

In an average, 40.32 % of the bands across all primers for all loci were found to be polymorphic in *P. sarana* stocks which were lower to that obtained in the Indian major carps - *Labeo rohita*, *Catla catla*, *Labeo calbasu* and *Cirrhinus mrigala* (45 %) [16]. In the Thai barb *B. gonionotus*, the value, however, was only 20.51 % which was much lower than the indigenous stocks.

The present study indicates that comparatively higher level of genetic variation exists in the studied stocks of indigenous barb compared to Thai barb sample. The population differentiation value (PhiPT = 0.698) between the two species - *P. sarana* and *B. gonionotus* was found to be highly significant. This value is much higher than that obtained between *Tor tor* and *Tor putitora* (0.264; P<0.001) by Ghosh and Alam [20] using RAPD marker. However, the population differentiation value (PhiPT = 0.09) was found to be insignificant among the indigenous barb stocks.
The mean observed number of alleles across all primers for all loci was 1.6000 ± 0.4963 in *P. sarana* and 1.2444 ± 0.3857 in *B. gonionotus*. The number of alleles per locus is also a measure of gene diversity. The maximum observed number of alleles in a stock reflects a much better genetic condition of this stock over the others. The mean effective number of alleles across all primers for all loci was 1.2822 ± 0.3314 in *P. sarana* and 1.1955 ± 0.3355 in *B. gonionotus*. The effective number of alleles functions as another measure of gene diversity. This measure is the number of equally frequent alleles that would take to achieve a given level of gene diversity. The more are the effective number of alleles the higher is the level of gene diversity. At the maximum gene diversity, the effective number of alleles will be the same as the actual number of alleles. The gene diversity was found to be higher in *P. sarana* (0.1748 ± 0.1823) than in *B. gonionotus* (0.1063 ± 0.1763).

It is shown that the similarity index provides upwardly biased estimates of stock homozygosity but nearly unbiased estimates of the average identity-in-state for random pairs of individuals [17]. In the present study, band-sharing based intra-stock similarity indices of the indigenous barb and Thai barb were 89.57% and 94.26%, respectively. Similar results were also

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observed in different finfishes like *L. calbasu* (93%) [19] and *L. rohita* (94.88%) [6]. This implies that individuals within each stock are genetically more similar to each other, as is expected, than to individuals from all other stocks [21].

Nei's [13] original measures of genetic identity (I) and genetic distance (D) were used to evaluate the genetic difference and relatedness among *P. sarana* and *B. gonionotus*. It considers a variety of parameters to measure the genetic distance. A high level of genetic identity (0.9699) was found among the local barb stocks and, as expected, the Thai barb showed lower pair-wise genetic identity with the local barb stocks. The genetic identity value obtained in the present study is within the range of genetic identity reported in eight killifish (*Aphanius*) species and sub-species [22]. Among the local barb stocks, the highest genetic distance (0.0450) was found between the Mogra river and hatchery stocks. The pair-wise genetic distances value between the Thai and the indigenous barb stocks are much higher (0.9442, 1.0023, 1.0418) compared to the pairs of the local barb stocks. Mohindra *et al.* [23] observed genetic distance among five species of mahseer ranging from 0.0944 to 0.5696. The UPGMA dendrogram based on Nei's [12] original measures of genetic distance (D) resulted in two major distinct clusters. As expected, the Thai barb alone made one cluster, and the remaining three local barb stocks made another cluster.

RAPD markers have been proved as effective tools to genetically characterize any species. Using five primers and 20 fish from each of the four stocks, the present study revealed a remarkable level of intra-specific genetic variability in *P. sarana* though it is considered as a critically endangered species in Bangladesh. This study would be useful as the baseline information of *P. sarana* and *B. gonionotus* for further study. In the present study, we found higher level of genetic variations in *P. sarana* compared to *B. gonionotus.* Primer OPA 17 can successfully be used to differentiate the two species as it gives consistent bands of different sizes in *P. sarana* and *B. gonionotus*. The species-diagnostic bands can be isolated and sequenced to develop species-specific DNA markers for the two species. Knowledge of genetic structure of the major wild stocks and captive hatchery stocks is helpful for management of the stocks in order to maintain their genetic quality.

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